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Long-Lasting Cell-Mediated Immunity Induced by a Live *Francisella tularensis* Vaccine

A. TÄRNVIK,[1]* M.-L. LÖFGREN,[2] S. LÖFGREN,[1] G. SANDSTRÖM,[3] AND H. WOLF-WATZ[3]

Departments of Clinical Bacteriology[1] and Ecologic Zoology,[2] University of Umeå, S-901 85 Umeå, and National Defence Research Institute (FOA 4), S-901 82 Umeå,[3] Sweden

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The lymphocyte stimulation test was used to estimate specific cell-mediated immunity after vaccination with the live vaccine strain of *Francisella tularensis*. Nonvaccinated individuals and individuals vaccinated 1, 5 to 6, 7 to 8, and 9 years previously were tested. Lymphocytes from most vaccinees responded to an antigen preparation of the vaccine strain, and those vaccinated 9 years before testing responded to a similar extent as did vaccinees in the other groups. A new technique was developed to study the presence of T lymphocytes among the stimulated cells. Stimulated cells were allowed to incorporate [^{14}C]thymidine and were then fractionated into T and non-T lymphocytes. Most of the incorporation appeared in the fraction containing T lymphocytes. The data indicate that cell-mediated immunity endures for at least 9 years after vaccination with the live *F. tularensis* vaccine.

Tularemia is a highly infectious bacterial disease caused by *Francisella tularensis*. Before 1960, few persons escaped illness if they continued to work with the organism (14, 20). In 1960 an attenuated strain of *F. tularensis*, called the live vaccine strain (LVS), was introduced at Fort Detrick, Md., for intracutaneous immunization (5) and was found to be protective insofar as the incidence of tularemia pneumonia was greatly reduced (2).

The duration of the protective effect of tularemia vaccination is unknown. In the Soviet Union a viable tularemia vaccine has been used in mass vaccinations since 1946 (for review see reference 15). Skin test reactivity and agglutinating serum antibodies have been found to persist for at least 5 years, which has been officially set as the vaccination interval for areas in the Soviet Union in which the disease is endemic (13). Revaccination at intervals as short as 2 to 3 years has led to marked general reactions with lymphadenitis, fever, and loss of working capacity (15).

A laboratory test for specific antimicrobial resistance against tularemia should be based on cell-mediated immunity (4, 10, 12). A suitable in vitro test for cell-mediated immunity in humans seems to be the lymphocyte stimulation test. Lymphocytes from tularemia-vaccinated individuals have been found to respond to protein antigen of the vaccine bacteria, whereas lymphocytes from nonvaccinated individuals responded poorly or not at all (9, 16, 18). Most of the blast cells formed at stimulation had the surface characteristics of T lymphocytes, since they formed rosettes with sheep erythrocytes (17).

The lymphocyte reactivity towards *F. tularensis* antigen was studied 1.5 (9) and 2 (19) years after LVS vaccination and found to persist for this period. We have now performed the test on individuals vaccinated up to 9 years before testing. Furthermore, a new test has been developed to confirm that cells stimulated have the characteristics of T lymphocytes.

MATERIALS AND METHODS

Tularemia vaccine. The LVS of *F. tularensis* was supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md., and was used as specified in the instructions given by the manufacturer.

Stimulating agents. *Francisella* antigen was prepared from *F. tularensis* LVS as previously described (16). It showed a specific reactivity in the lymphocyte stimulation test and the enzyme-linked immunosorbent assay (16). The antigen was suspended in RPMI 1640–*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (RPMI-HEPES; GIBCO Laboratories, Grand Island, N.Y.) at a density of 1.0 or 0.1 μg/ml. Purified protein derivative of tuberculin (PPD; Statens Seruminstitut, Copenhagen, Denmark) was solubilized in RPMI-HEPES at a concentration of 10 μg/ml.

Blood donors. Blood samples were obtained from 10 individuals vaccinated with *F. tularensis* LVS 1 year previously, from 10, 9, and 9 individuals vaccinated 5 to 6, 7 to 8, and 9 years previously, respectively, and from 10 individuals who denied previous tularemia or tularemia vaccination. Not all individuals were tested by all parameters used.

Lymphocyte stimulation test. Lymphocytes were prepared from heparinized blood by centrifugation on Lymphoprep (Nyegaard A/S, Oslo, Norway) as described by Böyum (1), washed, and suspended at a density of 3×10^6 cells per ml in a culture medium containing 80% RPMI-HEPES and 20% pooled normal human serum. The medium contained 100 μg of gentamicin per ml. Lymphocyte cultures were established in Microtest II tissue culture plates (Becton Dickinson Labware, Oxnard, Calif.). The cultures contained 100 μl of lymphocyte suspension and 100 μl of RPMI-HEPES with or without stimulating agent. Three to six replicate cultures were prepared. The plates were closed with plastic film and incubated at 37°C for 6 days. The cultures were then pulsed with [^{14}C]thymidine and harvested as described by Hartzman et al. (6). The incorporation of [^{14}C]thymidine has been found to be optimal after about 6 days of incubation with antigen from *F. tularensis* (18).

Procedures undertaken to study the nature of stimulated cells. Lymphocytes (3×10^6) were incubated with *Francisella* antigen (0.5 μg/ml) in 2 ml of culture medium in tightly closed plastic tubes (12 by 75 mm) (no 2058; Becton Dickinson Labware) at 37°C for 6 days. Thereafter the

* Corresponding author.

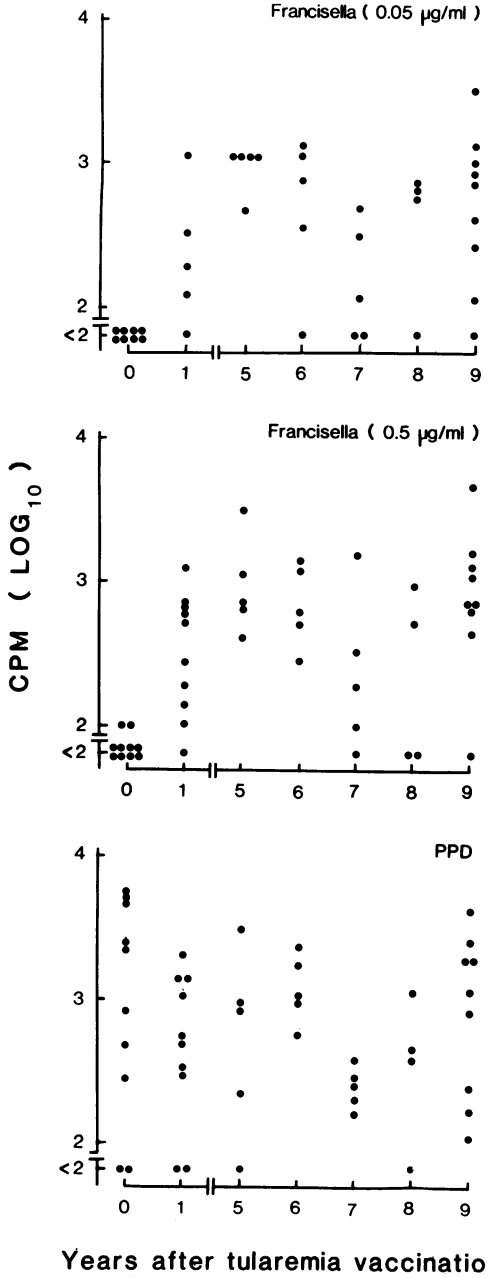

FIG. 1. Lymphocyte response to *Francisella* antigen and PPD (control antigen) before and at various intervals after vaccination with *F. tularensis* LVS.

lymphocytes were allowed to form rosettes with sheep erythrocytes (8) as previously described (17). Briefly, the lymphocytes from each tube were washed and suspended in 2 ml of fetal bovine serum. Sheep erythrocytes were washed and suspended in fetal bovine serum at a density of 150×10^6 cells per ml. A 1-ml volume of erythrocyte suspension was carefully pipetted on top of each lymphocyte suspension. After incubation overnight in an ice bath, the mixture of sheep erythrocytes and cultured cells was carefully suspended in 0.5 ml of fetal bovine serum and cytocentrifuged. From Giemsa-stained slides of each culture, 100 blast cells were examined for rosette formation.

A technique was developed to study the proportions of DNA synthesis induced in the T-lymphocyte and non-T-lymphocyte fractions of lymphocyte cultures. Lymphocytes were incubated in tubes for 6 days in the presence of *Francisella* antigen (0.5 or 0.05 μg/ml) and pulsed with [^{14}C]thymidine (0.4 μCi in 250 μl of RPMI-HEPES) for 6 h. They were mixed with sheep erythrocytes to allow rosette formation to occur, and the mixture was centrifuged on Lymphoprep as described previously (17). The cell suspension of the interphase between serum and Lymphoprep (non-T-lymphocyte fraction) was washed once in saline, distributed in microplates, collected onto glass fiber filters, and assayed for radioactivity (6). The T lymphocytes that had formed rosettes with sheep erythrocytes were collected at the bottom of the tube and assayed for radioactivity by the same procedure.

In control experiments [^{14}C]thymidine-pulsed lymphocytes were centrifuged on lymphoprep in the absence of erythrocytes; 90 ± 1.4% (mean± standard error of the mean of nine cultures) of the radioactivity appeared in the interphase.

Antibody assay. Serum was prepared and assayed for immunoglobulin M (IgM) and IgG antibodies toward the *Francisella* antigen by the enzyme-linked immunosorbent assay as previously described (16).

RESULTS

Lymphocyte response to *Francisella* antigen of individuals vaccinated 1 to 9 years before testing. Lymphocytes were incubated in the presence of *Francisella* antigen (0.5 or 0.05 μg/ml) or PPD (5 μg/ml). The incorporation of [^{14}C]thymidine into the DNA of the lymphocytes was measured by subtracting the incorporation induced in the absence of stimulating agent. Lymphocytes from nonvaccinated individuals responded to *Francisella* antigen with a low incorporation or did not respond at all, but responded with a high incorporation to PPD (Fig. 1). Lymphocytes from 31 of 38 tularemia-vaccinated individuals responded to *Francisella* antigen (0.5 μg/ml) with an incorporation higher than that induced in lymphocytes from any of the nonvaccinated individuals. The magnitude of [^{14}C]thymidine incorporation induced by *Francisella* antigen (0.5 or 0.05 μg/ml) into lymphocytes from individuals vaccinated 9 years before testing was about as high as that induced in lymphocytes from the other groups of vaccinees (Fig. 1).

Nature of stimulated cells. The presence of T lymphocytes among lymphocytes stimulated by *Francisella* antigen was investigated. Lymphocytes were obtained from four tularemia-vaccinated individuals. After incubation with *Francisella* antigen (0.5 μg/ml) for 6 days, the lymphocytes were allowed to form rosettes with sheep erythrocytes. The percentage of rosette-forming blast cells was calculated to estimate the proportion of T lymphoblasts among stimulated cells; 50 to 87% of the cells were rosette-forming.

A new technique was developed, by which it was confirmed that a great proportion of the *Francisella* antigen-induced lymphocyte stimulation occurred in T lymphocytes. Lymphocytes from individuals vaccinated 1, 7, and 9 years previously were incubated with *Francisella* antigen for 6 days, pulsed with [^{14}C]thymidine, and mixed with erythrocytes to allow rosette formation to occur. The mixtures were then separated on Lymphoprep, and the interphase (non-T-lymphocyte fraction) and the bottom fraction (T-lymphocyte fraction) were recovered and assayed for radioactivity; values obtained in the absence of stimulating agent were subtracted. A total of 66 to 100% of the radioactivity was

TABLE 1. Proportion of newly synthesized DNA in T-cell and non-T-cell fractions of *Francisella* antigen-stimulated lymphocytes[a]

Subject no.	Time from vaccination to testing (years)	Test antigen	Dose (μg/ml)	Radioactivity[b]	
				T-lymphocyte fraction	Non-T-lymphocyte fraction
1	1	*Francisella* antigen	0.5	4.9 (87)	0.7 (13)
		Francisella antigen	0.05	2.3 (66)	1.2 (34)
		PPD	5	2.1 (87)	0.3 (13)
2	7	*Francisella* antigen	0.5	10.3 (86)	1.7 (14)
		Francisella antigen	0.05	7.8 (74)	2.7 (26)
		PPD	5	6.9 (95)	0.4 (5)
3	9	*Francisella* antigen	0.5	4.1 (93)	0.3 (7)
		Francisella antigen	0.05	1.7 (85)	0.3 (15)
		PPD	5	11.9 (98)	0.2 (2)
4	9	*Francisella* antigen	0.5	7.3 (100)	0.0 (0)
		Francisella antigen	0.05	6.4 (100)	0.0 (0)
		PPD	5	2.8 (100)	−0.1 (0)
5	9	*Francisella* antigen	0.5	6.7 (81)	1.6 (19)
		Francisella antigen	0.05	1.4 (78)	0.4 (22)
		PPD	5	12.3 (95)	0.7 (5)

[a] Lymphocytes from tularemia-vaccinated individuals were pulsed with [^{14}C]thymidine after 6 days of incubation with *Francisella* antigen, with PPD, or without antigen and thereafter fractionated into T and non-T lymphocytes.

[b] The incorporation of [^{14}C]thymidine into DNA was calculated as counts/min × 10^{-3} after subtraction of values obtained in the absence of antigen. Numbers in parentheses represent percent radioactivity in the two fractions.

recovered in the T-lymphocyte fraction (Table 1). Similar results were obtained with PPD, which is held to induce T-lymphocyte stimulation in sensitized individuals (7).

Antibody response to *Francisella* antigen of individuals vaccinated 1 to 9 years before testing. Serum specimens were tested by the enzyme-linked immunosorbent assay for the presence of IgG and IgM antibodies toward *Francisella* antigen. Individuals vaccinated 9 years before testing had IgG and IgM antibodies at about the same levels as those vaccinated 1 to 8 years before testing (Fig. 2). Nonvaccinated individuals lacked antibodies (Fig. 2).

No correlation was found between the magnitude of lymphocyte stimulation and the amount of IgM or IgG antibodies when 36 tularemia-vaccinated individuals were investigated (data not shown). This is compatible with the view that the two assays estimate different arms of the immune response against *F. tularensis*.

DISCUSSION

In tularemia the specific antimicrobial host resistance depends mainly on cell-mediated immunity (4, 10, 12). Previously reported data (16–19) has indicated that the lymphocyte stimulation test could be used to demonstrate cell-mediated immunity in tularemia-vaccinated individuals. First, most of the blast cells formed by *F. tularensis*-induced stimulation of a mononuclear leukocyte preparation had the characteristics of T cells, being able to form rosettes with sheep erythrocytes (17). In addition, purified T lymphocytes from the vaccinees responded in the test, whereas a B-lymphocyte-enriched cell preparation did not respond (17). Furthermore, lymphocyte reactivity and reactivity with serum antibody were found to be due to different molecular structures of *F. tularensis* (16), and no correlation was found in tularemia-vaccinated individuals between the magnitude of lymphocyte response and the level of serum antibody (18, 19). We have now developed a technique for estimation of DNA synthesis of the T-cell as well as the non-T-cell fraction of stimulated lymphocytes, and the results confirmed that the lymphocyte stimulation test mainly estimated T-lymphocyte reactivity.

According to the present data, cell-mediated immunity is still apparent 9 years after vaccination. Such a long-lasting cell-mediated immunity is in agreement with that shown for other intracellular microbial agents (11). Apart from a long life of T memory cells (11), it is possible that antigen from *F. tularensis* is deposited in the host for a long time after infection or vaccination and may so entertain immunization. This is somewhat supported by the occurrence of homologous IgM antibodies in serum 9 years after vaccination, as shown in this study, and 2.5 years after illness, as shown by others (3).

As in previous studies (9, 19) there was a wide variability in lymphocyte quantitative response among tularemia vaccinees. Lymphocytes from some individuals showed a low response; this has previously been found consistent when such individuals are tested during several months after vaccination (19). The wide variability among vaccinees to respond in the lymphocyte stimulation test is in accordance with the known failure of the LVS vaccine to prevent all tularemia infections. At Fort Detrick, 11 tularemia infections occurred during the decade after introduction of the vaccine. At least three of these infections occurred only 1 year after vaccination (2), supporting the assumption that there is an individual variability in response to the vaccine rather than a general time-dependent declining immunity. Altogether, it may be worthwhile to test high-risk employees after vaccination, independent of vaccination interval. It would be interesting to revaccinate poorly responding individuals and retest them for lymphocyte reactivity, thereby distinguishing between an occasional and a consistent failure to respond to the vaccine.

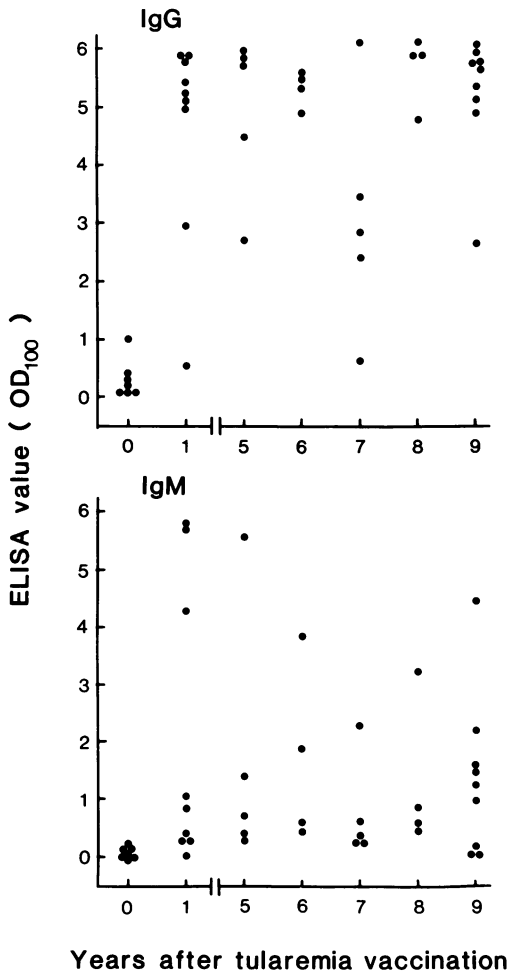

FIG. 2. IgG and IgM responses to *Francisella* antigen (1 μg/ml) before and at various intervals after vaccination with *F. tularensis* LVS.

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